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Cholesterol homeostasis in T cells. Methyl-β-cyclodextrin treatment results in equal loss of cholesterol from Triton X-100 soluble and insoluble fractions

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ABSTRACT

Methyl-β-cyclodextrin (MBCD) is frequently used to acutely deplete cells of cholesterol. A widespread assumption is that MBCD preferentially targets cholesterol in lipid rafts and that sensitivity to MBCD is proof of lipid raft involvement in a cellular process. To analyse any MBCD preference systematically, progressive cholesterol depletion of Jurkat T cells was performed using MBCD and [^{3}H]-cholesterol. It was found that at 37 °C, MBCD extracts similar proportions of cholesterol from the Triton X-100 resistant (lipid raft enriched) as it does from other cellular fractions and that the cells rapidly reestablish the relative differences in cholesterol concentration between different compartments. Moreover, cells restore the cholesterol level in the plasma membrane by mobilising cholesterol from intracellular cholesterol stores. Interestingly, mere incubation at 0 °C caused a loss of plasma membrane cholesterol with a concomitant increase in cholesteryl esters and adiposomes. Moreover, only 35% of total cholesterol could be extracted by MBCD at 0 °C and was accompanied by a complete loss of plasma membrane and endocytotic recycling centre filipin staining. This study clearly shows that MBCD does not specifically extract cholesterol from any cellular fraction, that cholesterol redistributes upon temperature changes and that intracellular cholesterol stores can be used to replenish plasma membrane cholesterol.

1. Introduction

Cholesterol is the most abundant sterol in mammalian membranes and its levels are under rigid control by complex mechanisms [1]. Due to its small polar headgroup and large hydrophobic moiety, cholesterol associates with phospholipids in order to avoid unfavourable exposure to water [2]. The plasma membrane of mammalian cells contains several types of nanodomains of which lipid rafts, in themselves a mixture of small domains with similar properties, are thought to form by the self-aggregation of cholesterol and sphingolipids [3]. Lipid rafts are believed to exist in a liquid ordered (lo) like state that resembles both the liquid disordered state, in that the lipids are fluid, and the gel state, in that the lipids are highly organised. Lipid rafts have become an attractive explanation for processes as diverse as the differential sorting of proteins in epithelial cells, immunological signalling and the entry of pathogens into host cells.

Cholesterol depletion is a popular tool in lipid raft research, based on the idea that cholesterol is an absolute requirement for lipid raft integrity and that its depletion leads to lipid raft dispersion, i. e. if a cellular process is affected by cholesterol depletion it is assumed that lipid rafts are involved. Various methods to lower cholesterol levels have been employed; the culture of cells in the absence of exogenous cholesterol [4], inhibition of cholesterol biosynthesis by statins [5], oxidation of cholesterol [6] and the use of cholesterol binding agents such as digitonin, filipin and saponin [7,8]. The most commonly used method is acute cholesterol depletion using methyl-β-cyclodextrin (MBCD). Unlike the cholesterol binding agents mentioned above that incorporate into membranes, MBCD has a central cavity able to form a 2:1 complex with cholesterol [9,10] and it acts strictly at the membrane surface.

It is a common misconception that MBCD preferentially extracts cholesterol from lipid rafts and it even gets referred to as a lipid raft inhibitor [11–14]. However, cholesterol is not confined to lipid rafts and it is debatable whether it is even enriched in these domains. The phase diagram of the lipid mixture cholesterol:sphingomyelin: dioleylphosphatidyl-choline demonstrates that the difference in cholesterol concentration between lo-domains and ld-domains is quite small [15]. A recent study showing that dihydroergosterol (DHE) is evenly distributed in the plasma membrane of macrophages and hepatoma cells suggests that there is no big difference in cholesterol concentration in different membrane domains also in cells [16]. There is no compelling reason why MBCD should specifically extract cholesterol from lipid rafts. On the contrary, it seems likely that cholesterol would actually be preferentially removed from other areas in the plasma membrane, where the lipids are less tightly packed.

The bulk of free cellular cholesterol, 65–90%, has been reported to reside in the plasma membrane, based on subcellular fractionation or cholesterol oxidase accessibility (reviewed in [17]). However, a study with the fluorescent cholesterol analogue DHE has demonstrated that a substantial cholesterol fraction, 35%, is found in the endocytic recycling compartment (ERC) [18]. In addition, ER membranes contain about

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1.5% cholesterol whereas in the plasma membrane [19], about 30–40% of the lipids are cholesterol. The ratio between the plasma membrane and intracellular membranes differs considerably between cell types, but typically the plasma membrane makes up no more than 5% of the total cellular membrane and the ER accounts for a much larger percentage [20,21]. Clearly this is incompatible with the bulk of the free cholesterol being in the plasma membrane and there is a need for new methods to reinvestigate this issue.

In this study, the distribution of cholesterol in Jurkat T cells subjected to progressive cholesterol extraction with MBCD was addressed. The results presented stress that cells maintain differences in cholesterol levels between cellular compartments by redistributing their remaining cholesterol. Furthermore, upon cold stress cells rearrange their cholesterol which can be quantified to estimate the proportion of cholesterol present in the plasma membrane at physiological temperature.

2. Materials and methods

2.1. Tissue culture

E6.1 Jurkat T cells from ATCC were cultured in RPMI 1640 medium supplemented with 5% heat-inactivated FCS, 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin (complete medium) in a humidified incubator under 5% CO_2.

2.2. Materials

CT-B-Alexa 594, anti-rabbit-Alexa 488 and avidin-Alexa 488 were from Molecular Probes (Invitrogen, Carlsbad, CA). Anti-transferrin receptor (TfR) was from MorphoSys (Kingston, NH). [^{3}H]-cholesterol was from Amersham Biosciences (GE Healthcare, Bucks, UK), EN^{3}HANCE spray from Perkin Elmer (Waltham, MA) and TLC-plates from Merck KGaA (Darmstadt, Germany). Sulfo-NHS biotin was from Pierce biotechnologies (Rockford, IL). Unless otherwise stated, chemicals were from Sigma (St Louis, MO).

2.3. Labelling Jurkat T cells with [^{3}H]-cholesterol

2×10^6 cells were washed twice with serum-free medium. Cells were grown for up to 40 h in the presence of 10 μCi [^{3}H]-cholesterol (specific activity 40–42 Ci/mmol) in complete RPMI except that the FCS concentration was 2.5%. Cell aliquots were subjected to liquid scintillation counting after washing in serum-free medium twice. Cells were lysed in chloroform:methanol:water (1:1:0.3 v/v) in which two phases were induced by changing it to chloroform:methanol:water (3:2:1 v/v). The organic phase was evaporated, the residue dissolved in a small volume of chloroform and then loaded on a silica column. Neutral lipids were eluted with chloroform and analysed by TLC on Silica plates developed with chloroform:acetone (49:1 v/v) or by reversed phase HPLC on a Hewlett Packard Hypersil ODS column (pore size 3 μm) using a linear gradient from methanol: water (9:1) to methanol:2-propanol:*n*-hexane (2:1:1). The radioactivity of the eluate was monitored by a radioactivity flow detector (Radiometric Instruments, Tampa, FL). The TLC plate was sprayed with EN^{3}HANCE and the plate was exposed to photographic film for 48 h at −80 °C.

2.4. Determination of cholesterol and cholesteryl esters

2×10^6 cells were washed and extracted as above. The water phase was then extracted with chloroform:methanol (3:1 v:v). The organic phases were pooled, evaporated and the tubes placed at 110 °C 20 min. The residue was dissolved in 200 μl assay buffer (0.1 M potassium phosphate pH 7.4, 50 mM NaCl, 5 mM cholic acid and 0.1% (w/v) Triton X-100 (TX)) and incubated at 37 °C 2 h. Cholesterol and cholesteryl esters were determined using 10-acetyl-3,7-dihydroxyphenoxazine (Amplex Red) [22,23]. 10 μl aliquots were incubated with 2 U/ml HRP and cholesterol oxidase, 300 μM Amplex red ±0.2 U/ml cholesterol esterase at 37 °C. Fluorescence was read after 150 min with excitation at 544 nm and emission at 590 nm in a Fluoroskan II (Labsystems, GMI, Ramsey, MN).

2.5. Depletion of cellular cholesterol

Labelled cells were washed twice with serum-free medium and mixed with non-labelled cells at a ratio of 2:3. Cells were left to equilibrate for 45 min at 37 °C in complete RPMI supplemented with 2% BSA and were then washed twice with serum-free medium. MBCD was dissolved freshly before each experiment and the cell density was kept constant at 10×10^6 cells/ml. Cells at 37 °C were treated with MBCD in the 0.5–15 mM range. At the indicated times, the cells were pelleted by brief centrifugation and the MBCD-containing supernatant was transferred to a fresh tube. The cell pellet was resuspended in PBS and aliquots from both fractions were subjected to scintillation counting. Two protocols resulting in 10%, 20%, 30%, 40% and 50% cholesterol extraction at 37 °C were used for further experiments. In the first protocol, cholesterol was extracted with 2.5 mM MBCD for 2.5, 5.5, 9 and 14.5 min or with 5 mM MBCD for 5 min. In the second protocol, cholesterol was extracted for 15 min with 0.5, 1, 1.2, 2.5 and 3 mM, respectively. Cells at 0 °C were treated with MBCD in the 30–120 mM range.

2.6. Drug treatment

Cells were treated with the HMG-CoA reductase inhibitor simvastatin at 20 μM at a cell density of 2×10^6/ml for 15 min prior to and during MBCD extraction. Simvastatin was also present during the recovery period.

2.7. Cellular fractionation

Labelled cells were mixed with non-labelled cells at a ratio of 2:23 and washed twice with serum-free medium. Cells were treated with MBCD to achieve the desired cholesterol depletion after which they were pelleted and washed in PBS. Triton X-100 detergent resistant membranes (TX-DRMs) were prepared as described previously from 50×10^6 cells lysed for 15 min on ice in 1 ml MNE (25 mM MES pH 6.5, 150 mM NaCl, 2 mM EDTA) containing 1% TX, protease inhibitors (5 μg/ml each of antipain, leupeptin, chymostatin and pepstatin and 1 mM PMSF), 5 mM NaF and 1 mM Na_3VO_4. [24]. The sucrose density gradient was made up of 2 ml 40% sucrose, 2 ml 30% sucrose and 1 ml 5% sucrose, all in MNE. TX-DRMs were collected from the 5–30% sucrose interface and pelleted by centrifugation at 100 000 ×*g* for 1 h. The resulting TX-DRM pellet was rinsed and suspended in MNE as was the pellet from the sucrose density gradient tube. The bottom 1.5 ml of the gradient was named the TX-soluble fraction and the 1.5 ml above the intermediate fraction. The resuspended pellets and aliquots of the other fractions were subjected to liquid scintillation counting. In another set of experiments, ten fractions of 500 μl were collected from the bottom of the tube were subjected to liquid scintillation counting.

2.8. FACS analysis

Control cells and cells treated with MBCD were stained with 2.5 μg/ml CT-B-Alexa 594 in PBS containing 2% BSA for 30 min at rt at a cell density of 5×10^6/ml. The cells were analysed on a FACSCalibur (BD Biosciences, San José, CA) with excitation at 595 nm and a 660/8 emission filter. Cells depleted of 50% cholesterol were left to recover in serum-free media for 40 min at 37 °C after which they were fixed for 15 min in 4% PFA. The cells were stained with filipin and washed as above and analyzed on a LSRII (BD Biosciences, San José, CA) with excitation at 358 nm and a 461/50 nm excitation filter as were untreated cells. 10 000 cells from each sample were analysed.

2.9. Filipin staining

For fluorescence microscopy experiments, cells were attached to TESPA-coated coverslips by incubation at 37 °C for 10 min or on ice for 45 min (2.5×10^5 cells/coverslip). Cholesterol extractions at the respective temperatures were performed at the same cell to MBCD ratios as above. Cells were fixed in 4% paraformaldehyde/PBS either at 37 °C for 15 min or at 0 °C for 60 min. A filipin stock of 20 mg/ml DMSO was stored under argon gas at −20 °C. Cells were stained with 50 μl of 50–250 μg/ml filipin at rt for 60 min. Cells were washed three times with PBS and mounted in AF1 (Citifluor Ltd, UK).

2.10. Surface biotinylation

5×10^6 cells were washed with ice-cold PBS, resuspended in 1 mg/ml sulfo-NHS biotin/PBS and incubated at 0 °C for 30 min. The cells were then washed in 100 mM glycine/PBS three times and attached to TESPA-coated coverslips (2.5×10^5 cells/coverslip). The cells were incubated at 37 °C for 10 min or at 0 °C for 20 min and fixed in 4% paraformaldehyde/PBS at the respective temperatures. The cells were stained with 10 μg/ml avidin-Alexa 488 for 30 min at rt.

2.11. Cell viability

Cell viability was assessed by trypan blue exclusion. For each population 300 cells were counted and each experiment was repeated three times.

2.12. Imaging

Wide-field fluorescence microscopy was performed with a Zeiss Axiovert 200 M microscope (Carl Zeiss MicroImaging GmbH, Göttingen, Germany) equipped with a AxioCam MRm camera. A 63X oil objective lens (NA 1.4) and DG4 (Sutter Instrument, Novato, CA) with excitation filters 350/50, 480/20 or 565/25 and emission filters 460/50, 525/50 or 645/75 were used for filipin, Alexa-488 and Nile red, respectively. DG4 excitation enabled the acquisition of high quality images of filipin, which is prone to rapid photobleaching when excited with most light sources. To avoid bleaching affecting the image analysis, focus was adjusted under transmitted light and filipin images immediately acquired when the excitation source was turned on. Imaging was performed at the equatorial plane of the cells. Each experiment was repeated at least twice and more than 100 cells meeting the criteria were visually examined from each population. The images were acquired blindly to minimise operator bias. Displayed images were prepared using Adobe Photoshop 7.1 software.

2.13. Estimation of filipin in the plasma membrane

The plasma membrane was delineated manually with sequentially marked points that were joined automatically [25]. Once delineated, the mean fluorescence intensity

per pixel was calculated. The software was built around a Semper6w kernel (Synoptics Ltd, Cambridge, UK). Cells selected for image analysis met the criteria of having no near neighbours and nuclei clearly discernable by phase contrast. Imaging was performed at the equatorial plane of the cells.

2.14. Quantitation of adiposomes

Cells were attached to coverslips, incubated and fixed at 0 or 37 °C as above were stained with 50 μl of 100 μg/ml Nile red for 15 at rt. Z-sections of 0.25 μm were acquired throughout the cells. The number of adiposomes in a cell was obtained by sequentially examining the Z series and noting the first appearance of each adiposome.

3. Results

3.1. Labelling of Jurkat T cells with [^{3}H]-cholesterol

Jurkat T cells were incubated with [^{3}H]-cholesterol at a range of concentrations for different times in the presence of various concentrations of FCS. Incubation for 40 h resulted in the highest level of labelling, assessed by scintillation counting after repeated washing of the cells (not shown). Neutral lipid extracts from cells were analyzed by both TLC and HPLC (Supplemental Fig. 1) which showed that [^{3}H]-cholesterol was not metabolized after labelling. Notably, [^{3}H]-cholesterol incorporation in to cholesteryl esters was minimal most likely due to the slow incorporation of plasma membrane cholesterol in to cholesteryl esters [26].

3.2. Cholesterol depletion by MBCD at 37 °C

Jurkat T cells labelled with [^{3}H]-cholesterol were treated with fresh MBCD in serum-free HEPES-buffered RPMI at various concentrations for different times. After brief centrifugation, aliquots from the cell pellets and supernatants were subjected to scintillation counting. Addition of a large excess of MBCD to the cell suspension prior to centrifugation did not change the distribution of label nor did the supernatant when transferred to 0 °C give rise to a pellet of precipitated [^{3}H]-cholesterol-MBCD upon centrifugation. This indicates that the label in the pellet was unextracted [^{3}H]-cholesterol [27].

At all concentrations of MBCD tested, the cholesterol was extracted at a single rate that reached a plateau specific for each MBCD concentration. The plateau is likely to be at the equilibrium between free and cholesterol-bound MBCD in water solution. At equilibrium no more than 1.5% of the MBCD was bound to cholesterol assuming that 2:1 MBCD to cholesterol complexes were formed (see Appendix for calculations). This does not necessarily mean that all the non-cholesterol-bound MBCD was free since MBCD can bind a variety of amphiphiles [10].

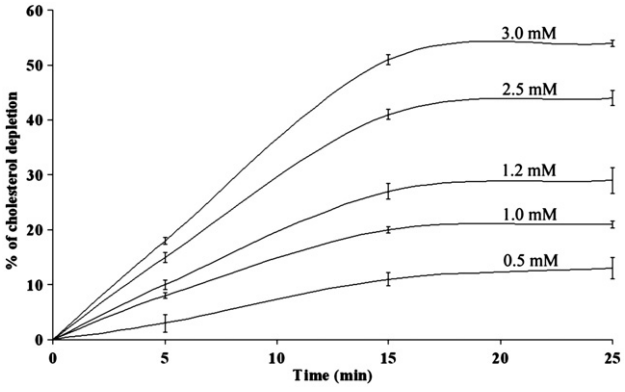

Fig. 1. Cholesterol extraction from Jurkat T cells by MBCD at 37 °C. 10×10^6 Jurkat T cells/ml labelled with [^{3}H]-cholesterol were treated with MBCD in serum-free RPMI medium at 37 °C. At the indicated times, the cells were pelleted by a brief centrifugation and the MBCD-containing supernatant was transferred to a fresh tube. The cell pellet was resuspended in PBS and aliquots from both fractions were subjected to scintillation counting. Data shown are means ± SD, $n = 3$.

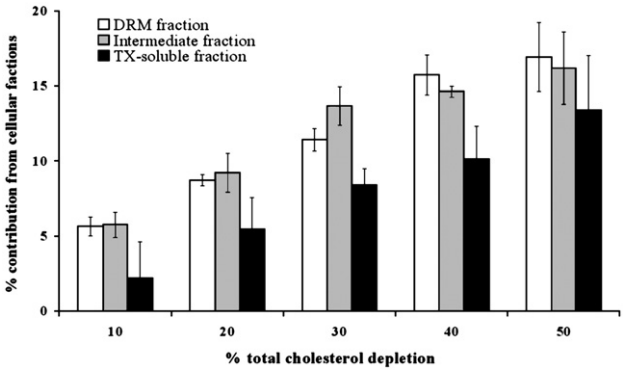

Fig. 2. Cholesterol extraction from cellular fractions by MBCD at 37 °C. Cells labelled with [^{3}H]-cholesterol were treated with MBCD at 37 °C to achieve the desired cholesterol depletion after which they were pelleted and washed in PBS. TX-DRMs, the TX-soluble and the intermediate fractions were prepared as described under experimental procedures. The resuspended pellets and aliquots of the other fractions were subjected to liquid scintillation counting. Data shown are means ± SD, $n = 3$.

Viability of cells was assessed by trypan blue staining. Cell death was correlated with the extent of cholesterol extraction and 91% of the cells were still viable after 50% cholesterol depletion (Supplemental Table 1). More than 50% depletion resulted in substantial cell death and was therefore avoided in further experiments. Conditions were standardised for the progressive depletion of roughly 10%, 20%, 30%, 40% and 50% cholesterol. In the first protocol, cholesterol was extracted with 2.5 mM MBCD for 2.5, 5.5, 9 and 14.5 min or with 5 mM MBCD for 5 min. In the second protocol, cholesterol was extracted for 15 min with 0.5, 1, 1.2, 2.5 and 3 mM, respectively (Fig. 1). Since depletion at equilibrium is expected to cause less variation between experiments, the second protocol was preferred although the two protocols produced interchangeable results. GM1 was lost from the cells upon MBCD treatment but to a lesser extent than was cholesterol. When the cells had lost 50% of their total cholesterol, they had lost 23% of their GM1 assessed by FACS analysis.

3.3. MBCD treatment at 37 °C is not specific for cholesterol from any cellular compartment

In many studies it is assumed that MBCD easily removes cholesterol from lipid rafts. However, from what cellular fraction cholesterol removal *de facto* takes place is rarely considered. To address this progressive cholesterol depletion, performed as described above, was followed by lysis in 1% TX on ice and the lysate was subjected to equilibrium centrifugation on a sucrose gradient [24]. The lipid raft enriched TX-DRMs, the TX-soluble containing fully solubilised cellular components and the intermediate fraction containing not fully solubilised cell material with a higher protein to lipid ratio than TX-DRMs were defined as described under materials and methods. Minimal amounts of cholesterol were recovered in the pellet and the fraction above the TX-DRMs, which justified disregarding these fractions in further analyses. Lipid raft cholesterol comprised 27% of total and the remaining 73% was distributed between the intermediate and TX-soluble fractions. The analysis revealed that cholesterol is extracted from all cellular fractions, i.e. not specifically from TX-DRMs (Fig. 2). Similar proportions were extracted from TX-DRMs and the intermediate fraction. To illustrate the importance on how TX-DRMs are prepared for the partitioning of cholesterol, a less rigorous protocol was also applied [28]. The amount of cholesterol in the different fractions clearly varies depending on what lysis conditions are used (Supplemental Fig. 2). The lower TX to cell ratio resulted in a much larger portion of total cholesterol in the TX-DRM fraction. It has been demonstrated that this method does not result in a lipid raft enriched DRM fraction [29]. Misleadlingly, MBCD may seem to have a preference

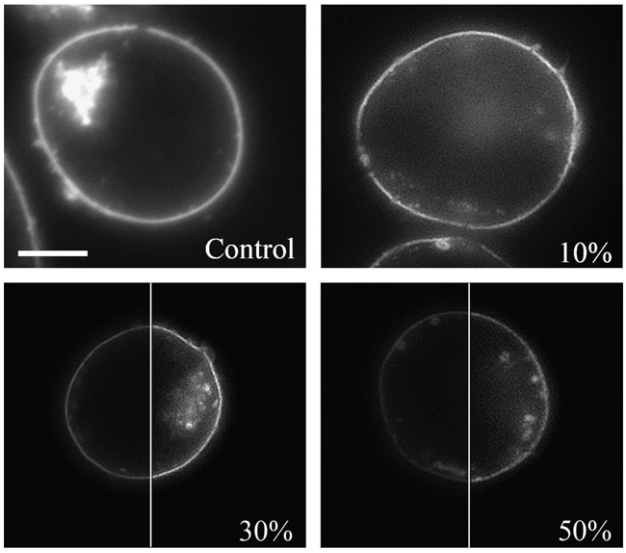

Fig. 3. Filipin staining of cells treated with MBCD at 37 °C. Jurkat T cells with 10%, 30% or 50% cholesterol removed by MBCD treatment at 37 °C. The cells were fixed in 4% PFA/PBS for 15 min at 37 °C and stained with filipin at rt. Cells shown are representative of six experiments. The right half of the two lower images has been contrast stretched to show detail. Scalebar 5 μm.

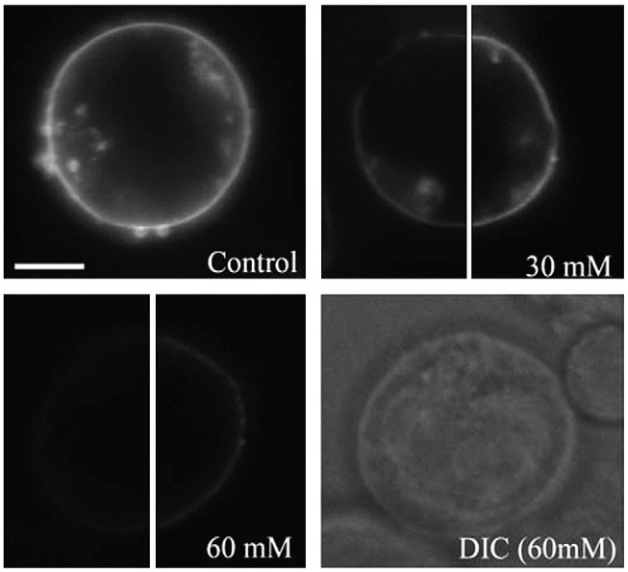

Fig. 5. Filipin staining of cells treated with MBCD at 0 °C. Jurkat T cells were treated with of 30 or 60 mM MBCD at 0 °C. The cells were fixed in 4% PFA/PBS for 60 min at 0 °C and stained with filipin at rt. Cells shown are representative of five experiments. The right half of the 30 and 60 mM images has been contrast stretched to show detail. DIC = differential interference contrast. Scalebar 5 μm.

for lipid raft cholesterol if the resulting TX-DRMs are equated with lipid rafts.

Filipin is a polyene antibiotic that interacts with cholesterol but not with cholesteryl esters [30]. In cells fixed at 37 °C the bulk of the staining was found in the plasma membrane (Fig. 3). This is in line with the cholesterol concentration being higher in the plasma membrane than other cellular membranes. Some staining could also be detected in the ERC, a perinuclear compartment that stained positive for the TfR (not shown). The permeability of the fixed cells to filipin was variable resulting in different intensities of the ERC staining. Permeabilisation with detergents was however ruled out since it clearly caused redistribution of plasma membrane cholesterol. Upon MBCD treatment the filipin staining in the plasma membrane was reduced and to a smaller extent so was the ERC filipin staining. When 50% of the total cholesterol had been extracted, filipin staining in the plasma membrane was very weak but clearly discernible when the image was contrast stretched (Fig. 3).

3.4. MBCD has a bias for TX-DRM cholesterol at 0 °C

Upon incubation at 0 °C, the plasma membrane of T cells contains domains enriched in signalling proteins that are visible by conventional light microscopy whereas at 37 °C it does not [24]. These domains are probably aggregated lipid rafts due to their enriched GM1 and Lck content. To investigate the possibility that cholesterol in aggregated lipid rafts is more difficult to extract than cholesterol in dispersed lipid rafts, MBCD extraction was performed at 0 °C. As expected, cholesterol depletion was less efficient at 0 °C than at 37 °C and there was a clear limit on how much cholesterol could be extracted at 0 °C. Even at a MBCD concentration of 120 mM, no more than 35% cholesterol could be extracted from the cells (Fig. 4). Considering that no label was incorporated in to cholesteryl esters and that they account for about 10% of total cholesterol in Jurkat T cells grown under these conditions, this number is likely to be a small overestimate of the amount endogenous cholesterol extracted. The 35% could correspond to the plasma membrane cholesterol since endocytosis and other vesicular transport

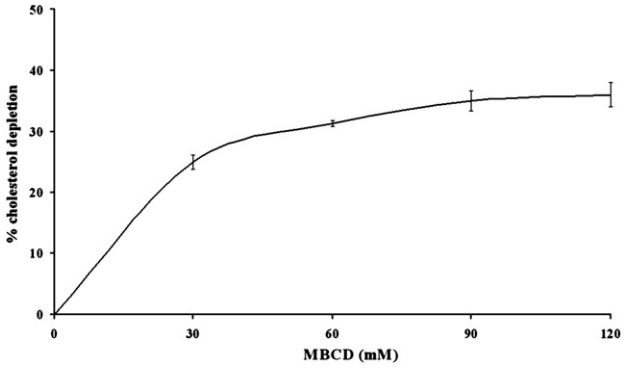

Fig. 4. Cholesterol extraction from Jurkat T cells by MBCD at 0 °C. 10×10^6 Jurkat T cells/ml labelled with [^{3}H]-cholesterol were treated with MBCD in serum-free RPMI medium at 0 °C for 45 min. The cells were pelleted by a brief centrifugation and the MBCD-containing supernatant was transferred to a fresh tube. The cell pellet was resuspended in PBS and aliquots from both fractions were subjected to scintillation counting. Data shown are means ± SD, $n = 3$.

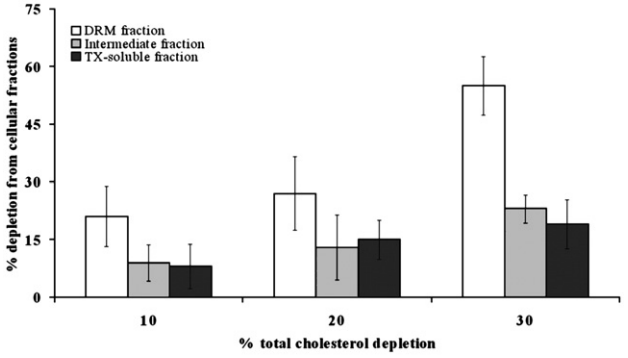

Fig. 6. Cholesterol extraction from cellular fractions by MBCD at 0 °C. Cells labelled with [^{3}H]-cholesterol were treated with MBCD at 0 °C to achieve the desired cholesterol depletion after which they were pelleted and washed in PBS. TX-DRMs, the TX-soluble and the intermediate fractions were prepared as described under experimental procedures. The resuspended pellets and aliquots of the other fractions were subjected to liquid scintillation counting. Data shown are means ± SD, $n = 3$.

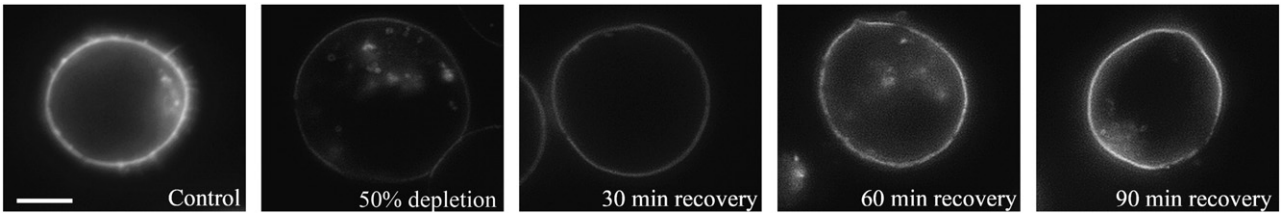

Fig. 7. Recovery of filipin staining after MBCD treatment at 37 °C. Cells treated with 50 μM simvastatin and control cells had 50% of their cholesterol extracted by MBCD at 37 °C. MBCD was washed out and the cells left in serum-free RPMI for 30, 60 or 90 min to recover. The cells were fixed in 4% PFA/PBS at 37 °C and stained with filipin at rt. Cells shown are representative of three experiments. Scalebar 5 μm.

are generally hindered at low temperatures. To confirm that endocytosis was impaired in our system, Jurkat T cells were surface biotinylated and the biotin tracked with avidin-Alexa-488. No endocytosis occurred at 0 °C (Supplemental Fig. 3).

Surprisingly, filipin staining revealed that cholesterol was extracted from both the plasma membrane and the ERC at 0 °C (Fig. 5). That no intracellular filipin staining could be achieved was confirmed in permeabilised cells (not shown). That MBCD entered the live cells was considered unlikely for two reasons. Firstly, there was no endocytosis and secondly the cells excluded trypan blue (Supplemental Table 2). This suggests that there is non-vesicular communication or even direct interaction between the ERC and the plasma membrane. No increase in the intensity of the filipin signal was observed upon either increasing the filipin to cholesterol ratio or the filipin staining time.

A protocol for the progressive depletion of 10%, 20% and 30% cholesterol at 0 °C was established by treating the cells with 30 mM MBCD for 10 and 20 min or 60 mM MBCD for 45 min, respectively. After depletion, the cells were subjected to lysis in 1% TX on ice followed by subfractionation as above. 55% the cholesterol was depleted from the TX-DRM fraction when 30% of total cholesterol was extracted (Fig. 6). This demonstrates that cholesterol extraction readily takes place from aggregated lipid rafts. The result is consistent with the majority of lipid rafts residing in the plasma membrane. At the maximal 35% cholesterol depletion at 0 °C we estimate by extrapolation that 70–80% of the TX-DRM cholesterol would have been removed suggesting this proportion corresponds to lipid rafts of plasma membrane and ERC origin.

3.5. Recovery of plasma membrane cholesterol after MBCD treatment

To investigate if plasma membrane cholesterol could be replenished after the removal of 50% of their cholesterol, the MBCD was washed out and cells were left to recover in media in the absence of FCS at 37 °C. The plasma membrane staining quickly recovered (Fig. 7). FACS analysis was used to get a measurement of fluorescence intensity of a large population. After 40 min recovery, the filipin staining was 60% that of control cells. However, FACS analysis does not provide spatial information about the fluorescence signal. To this end, the intensity of the plasma membrane staining was analysed. After a 60 min recovery period at 37 °C, the plasma membrane filipin staining was 75% that of control cells (Table 1) which is remarkable considering the cells had lost 50% of their total cholesterol. Preincubation of the cells with 20 μM simvastatin to inhibit HMG-CoA reductase and thus cholesterol biosynthesis and its presence during the MBCD treatment and recovery period did not alter the extent of the recovery. However, the presence of simvastatin resulted in a greater absolute loss of plasma membrane filipin staining. During recovery the number of adiposomes in the cells decreased suggesting that stored cholesteryl esters were being hydrolysed to supply cholesterol (Table 2).

When cells depleted of 30% of their cholesterol with 90 mM MBCD at 0 °C were transferred to 37 °C there was no recovery of filipin staining (not shown). It turned out that the cells died on transfer to 37 °C, having lost almost all filipin stainable cholesterol in their plasma membranes at 0 °C (Fig. 5 and Supplemental Table 2).

3.6. Cold stress affects cholesterol homeostasis and metabolism

Cells incubated and fixed at 0 °C prior to filipin staining appeared less bright than those kept and fixed at 37 °C. However, the difference between individual cells in the population was substantial. Therefore a blind analysis of fluorescence intensity in the plasma membrane of cells randomly picked was performed. This confirmed that the plasma membrane of cells incubated and fixed at 0 °C had about 73% of the intensity of cells kept and fixed at 37 °C (Table 3). That this difference

Table 1
Recovery of plasma membrane filipin staining

Population	Fluorescence intensity in plasma membrane pixels (relative values)	Recovery (%)
Control	100±3.1	
50% cholesterol depletion	33.5±1.5	
60 min recovery	75.1±3.5	224
50% cholesterol depletion+simvastatin	25.7±2.1	
60 min recovery+simvastatin	61.5±2.8	239

Jurkat T cells were depleted of 50% of their total cholesterol at 37 °C±20 μM simvastatin present from 15 min prior to MBCD treatment until the end of the 60 min recovery period. The cells were kept and fixed at 37 °C and stained with filipin at rt. The plasma membrane was delineated manually with sequentially marked points that were joined automatically and the total fluorescence intensity of the selected pixels calculated. The fluorescence intensity in the plasma membrane pixels of the cells fixed at 37 °C was set as 100. Data shown are cellular means±s.e.m., n=40.

Table 2
Temperature and recovery variation of the number of adiposomes

Temperature (°C)	Number of adiposomes
37	28.2±1.2
37, 50% cholesterol depletion	25.7±0.7
37, 60 min recovery	20.2±0.8
37, 90 min recovery	19.9±0.6
0	40.5±1.7

Jurkat T cells were depleted of 50% of their total cholesterol at 37 °C and left to recover for 60 or 90 min or incubated and fixed at the respective temperature. Cells were stained with Nile Red. Z-sections of 0.25 μm were acquired throughout the cells and adiposomes were counted. Data shown are means±s.e.m., n=60.

Table 3
Intensity of plasma membrane filipin staining

Temperature (°C)	Fluorescence intensity in plasma membrane pixels (relative values)
37	100±3.1
0	73±2.6

Jurkat T cells either kept and fixed at 37 °C or incubated for 45 min and fixed at 0 °C. The cells were stained with filipin at rt. The plasma membrane was delineated manually with sequentially marked points that were joined automatically and the mean fluorescence intensity of the selected pixels calculated. The fluorescence intensity in the plasma membrane pixels of the cells fixed at 37 °C was set as 100. Data shown are cellular means±s.e.m., n=44.

Table 4
Temperature dependence of the cholesteryl ester pool

Temperature (°C)	Cholesteryl esters (% of total cholesterol)	Increase (%)
37	9.42±0.75	
0	15.91±3.8	67.4±28

Jurkat T cells were washed and extracted with chloroform:methanol:water. After evaporation of the organic solvents, the residue was dissolved in assay buffer containing HRP, cholesterol oxidase, Amplex red and cholesterol esterase. Fluorescence was read after 150 min with excitation at 544 nm and emission at 590 nm. Data shown are means±SD, $n=4$. $p<0.025$ for a two tailed *t*-test.

was not due to an increase in cellular size upon incubation at 0 °C was confirmed by FACS analysis. The intensity of plasma membrane GM1-CT-B-Alexa 594 staining was the same in cells incubated and stained at 0 and 37 °C (not shown). A suspicion was that the displaced cholesterol was incorporated in to cholesteryl esters. To this end, the Amplex Red assay where cholesterol oxidase with or without cholesteryl esterase are used to assess the amount of free and total cholesterol respectively, was employed. In cells at 37 °C, cholesteryl esters were about 9% of total cholesterol (Table 4). After 45 min at 0 °C, the amount of cholesteryl esters had increased by a remarkable 67% comprising 16% of total cholesterol. Cholesteryl esters are stored in adiposomes. Jurkat T cells kept and fixed at 37 °C contained an average of 28 adiposomes with the number of adiposomes per cell differing substantially (Table 2). After 45 min incubation and subsequent fixation at 0 °C, the average cell contained 40 adiposomes; a 46% increase. Although size of small particles is difficult to assess from fluorescence microscopy images, the adiposomes from the cells incubated at 0 °C also appeared to be somewhat larger than those in cells kept at 37 °C (not shown). This demonstrates that cells respond to cold stress by reorganising their cholesterol.

4. Discussion

MBCD, which has a central hydrophobic cavity able to form a 2:1 complex with cholesterol [9,10], has become a popular research tool in the field of lipid rafts based on the idea that cholesterol is an absolute requirement for lipid raft integrity and that its depletion causes lipid raft dispersion. There is no compelling reason to believe that MBCD specifically extracts cholesterol from lipid rafts. On the contrary, it seems likely that cholesterol would actually be preferentially removed from other areas in the plasma membrane, where the lipids are presumed to be less tightly packed. In addition, cells are dynamic and there is a continuous interchange of material between cellular compartments. This study shows that MBCD at 37 °C extracts a similar proportion of cholesterol from all cellular compartments, which is in line with the cell striving to maintain any intracellular cholesterol gradients.

One biochemical definition of lipid rafts is their insolubility in non-ionic detergents at 4 °C — a procedure that generates DRMs. TX-DRMs are enriched in cholesterol, glycosphingolipids, sphingomyelin and saturated glycerophospholipids [31]. These lipids can by themselves form a lo-like state at 37 °C, supporting the raft hypothesis [32]. Although TX is more selective than other detergents and TX insolubility is a good indication of lipid raft partitioning, TX-DRMs are substantially aggregated structures not equivalent to *bona fide* biological entities [33,34]. Cholesterol depletion makes some components of lipid rafts sensitive to TX extraction. However, both lipid and protein markers of lipid rafts can still be found in TX-DRMs after cholesterol depletion [35]. An interpretation of the results at 37 °C would be that upon subcellular fractionation following detergent extraction, cholesterol always partitions in a similar way between the various fractions. However, this was not case when the MBCD treatment was performed at 0 °C, arguing that cells do not behave like model membranes where a phospholipids:sphingolipids:cholesterol (1:1:1) mixture is what remains insoluble when a wide range of lipid compositions are extracted with TX-100 at 4 °C [36,37]. The sphingolipid GM1 was also lost from the cells, but to a far lesser extent than was cholesterol, considering the relative abundance of the two lipids. MBCD has more than a thousand fold higher specificity for cholesterol than for GM1, which makes it likely that GM1 *de facto* was extracted by MBCD rather than being lost from the plasma membrane by other means. If there was a methodologically induced redistribution of cholesterol, it would be expected to produce a constant percentage of cholesterol or a constant cholesterol to sphingolipid ratio in the TX-DRMs, neither of which was observed.

It has been suggested that brief MBCD treatment is specific for lipid raft cholesterol [28]. However, the authors used a lower TX to cell ratio than required to achieve specificity for raft lipids [29] and it is well known that lower TX to cell ratio results in more material, both lipids and proteins, appearing in the DRM fraction [38] making these results questionable. Lipid raft research has been plagued by the uncontrolled use of various detergents under different solubilisation conditions. Notably, also in a recent review it is concluded that brief MBCD treatment or the use of low MBCD concentrations could make it specific for lipid raft cholesterol [39]. Our study demonstrates that neither of these conclusions is valid.

There is proportionality in the absorbance of filipin added to liposomes and their cholesterol content over a tenfold range of cholesterol concentration [30]. Since absorbance is related to emission it is valid to assume that a proportional relationship exits also for filipin-cholesterol complex fluorescence.

In model membranes, filipin as a probe for quantitative assessment of cholesterol content has a few drawbacks in that staining is sensitive to sphingomyelin to cholesterol ratio, labelling time and filipin to cholesterol ratio [40]. In the present study, no enhancement of the signal was observed in several different populations upon either increasing the filipin to cholesterol ratio or the filipin staining time suggesting that the staining intensity is proportional to cholesterol content, at least above the putative threshold concentration. In addition, the sensitivity to sphingomyelin content is minimised by using a filipin to cholesterol ratio from which the signal can not be improved if the ratio is increased. There is a threshold under which no staining is evident. For tissues this threshold is estimated to be around 5–7 mole % cholesterol [40].

At 0 °C no more than 35% of the cholesterol could be extracted with MBCD. Cholesterol flip-flops over the membrane on a time-scale orders of magnitude shorter than the MBCD-treatment time and extraction is therefore not limited to the outer leaflet of the plasma membrane [27,41]. As anticipated the plasma membrane lost all its filipin staining after MBCD treatment at 0 °C and to our surprise, so did the ERC. Since the cells excluded trypan blue it seems unlikely that MBCD entered the cells. This suggests that the ERC communicates with the plasma membrane via non-vesicular sterol transport [42] or that the ERC is physically connected to the plasma membrane. On the other hand, ER to plasma membrane cholesterol transport is abolished at temperatures below 15 °C, consistent with it being of vesicular nature [43]. Cholesterol extraction on ice could be used to put an upper limit on how much of a cell's cholesterol can be found in the plasma membrane. Showing absolutely no filipin staining, the plasma membrane and the ERC together contain about 35% of cellular total cholesterol which corresponds to 38.5% of free cholesterol. The lack of vesicular transport at 0 °C and that a longer MBCD incubation time did not lead to further cholesterol extraction (not shown), suggests that no other organelles contributed their cholesterol during the extraction. Moreover, the latter also strongly indicates that the plasma membrane and the ERC were completely depleted of cholesterol.

That most of the cholesterol at 0 °C was extracted from the TX-DRM fraction fits well with the concept that there is a progressive increase in lipid raft partitioning molecules along the secretory pathway [44]. Although ER cholesterol was of a concentration too low to be

stained by filipin, there is far more ER than plasma membrane in cells so ER cholesterol could account for a substantial portion of the total free cholesterol. Membranes of other intracellular organelles like mitochondria, lysosomes and Golgi also contain cholesterol [45]. We estimate that the plasma membrane and the ERC in Jurkat T cells contain about 38% of total free cellular cholesterol. Difficulties in reproducibly labelling the ERC with filipin, in the absence of cell permeabilisation, combined with the membrane rearranging effect of permeabilisation unfortunately excluded reliable quantification of the distribution of cholesterol between these two organelles. By visual assessment, we estimate that a larger fraction of this cholesterol is found in the plasma membrane than in the ERC, which is consistent with the faster of the two pools identified in cholesterol exchange studies being of plasma membrane origin although this was not the interpretation favoured in those studies [46,47]. The ERC of CHO cells has been reported to contain 35% of total free cellular cholesterol [18], but there might be cell type specific differences, or, since DHE-loading is likely to disturb the cholesterol balance and the plasma membrane cannot accommodate much excess cholesterol [48], a large portion may accumulate in the ERC as a cholesterol reserve.

The loss of plasma membrane cholesterol and concomitant increase in cholesteryl esters at 0 °C is remarkable in that the later far exceeds the normal capacity of ACAT which is around 0.1% plasma membrane cholesterol per hour [26]. The modest increase in ACAT occurring upon long-term storage of microsomes at 4 °C has been ascribed to the redistribution of cholesterol [49]. ACAT activity, at least of ACAT1, *in vivo* is limited by the supply of sterols (reviewed in [50]). It is possible that the cholesterol lost from the plasma membrane is transported to the ER compartment where ACAT resides, thus increasing the rate of cholesterol esterification but, considering kinetic effects, some non-enzymatic cold stress mechanism might also be in play. It is also conceivable that cholesterol esterase is inhibited at low temperature contributing to the increase in cholesteryl esters. That cholesterol is lost from the plasma membrane upon cold exposure might be a mechanism to maintain membrane fluidity since cholesterol and decreasing temperature both make the membrane less fluid [51]. How the lipid composition and fluidity of the plasma membrane is affected by short term storage at 0 °C is an issue deserving further attention. Moreover, routines involving storage of cells on ice should be avoided since this produces a cellular response likely to effect the interpretation of subsequent experiments.

Interestingly, despite having lost 50% of their cholesterol at 37 °C and most plasma membrane filipin staining the cells were still viable. This means that a high concentration of cholesterol in the plasma membrane is not needed to maintain the permeability barrier. Moreover, within an hour at 37 °C their plasma membrane cholesterol was restored to 75% of that of control cells. Considering that there were no lipoprotein particles in the medium and that inhibition of cholesterol biosynthesis did not slow down the recovery, we conclude that cholesterol from intracellular sources is readily transported to the plasma membrane and must account for a large portion of the cell's total free cholesterol. Moreover, considering that the number of adiposomes decreased during recovery of plasma membrane filipin staining we suggest that a substantial part of the cholesterol is coming from cholesteryl esters that get hydrolysed when the cell rapidly needs more cholesterol. Cells maximally depleted of cholesterol at 0 °C and then transferred to 37 °C for recovery were on the other hand immediately permeable to trypan blue although they had only lost only 35% of their cholesterol. This suggests that there is a crucial minimal level at which cholesterol needs to be maintained in the plasma membrane to avoid leakage. Sterol transport ensures this at 37 °C but not at 0 °C.

5. Conclusions

In summary, acute cholesterol depletion using MBCD is not specific for cholesterol in any cellular compartment due to sterol dynamics. Cholesterol lost from the plasma membrane is rapidly replenished from intracellular cholesterol stores. It follows that studies on the effect of reduced plasma membrane cholesterol must be conducted immediately after the MBCD treatment. Moreover, storage of T cells on ice not only causes membrane reorganisation and signalling [24] but also alters the cholesterol and cholesteryl ester distribution.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbamem.2008.02.010.

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